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13. ABSTRACT (Maximum 200 Words)

NF1 is characterized clinically by the development of plexiform and multiple cutaneous neurofibromas. There is no correlation between the numbers, size or prevalence of neurofibromas and the type of mutations in the NF1 gene, suggesting a role for genetic modifiers. Genetic polymorphism in mitochondria could cause variability in the observed tumor phenotype in NF1. Here, we analyzed somatic mitochondrial DNA mutations in cutaneous and plexiform neurofibromas to determine if certain mutations are found predominantly in tumors. We found somatic mutations in 9 of 18 plexiform neurofibromas and 9 of 19 cutaneous neurofibromas. All mutations detected were in the hypervariable D-loop region, where origin of replication and transcriptional regulators are located. Most mutations appeared to be homoplasmic in the tumors. In addition, pre-existing soamtic mtDNA mutations were detected in healthy skin of NF1 patients. Our analysis found that these pre-existing somatic mtDNA mutations accumulate in the tumor, suggetsing a selection for the mutated mitochondria in all cell types present in neurofibromas. In a second ongoing set of experiments we analyze the proportion of germ line mitochondrial DNA variants in a cohort of 500 NF1 patients with high numbers and low numbers of cutaneous neurofibromas.

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Introduction

Neurofibromatosis type 1 (NF1) is the most common inherited predisposition for tumor development. The predisposing mutation was localized to the NF1 gene coding for neurofibromin, a widely expressed 2818 amino acid protein with ras-GAP activity (1-3). Clinical features of NF1 include pigmentation abnormalities, multiple tumors, skeletal lesions and specific learning dysfunctions (4,5). Tumors in NF1 comprise dermal neurofibromas, peripheral nerve sheath tumors and their malignant derivatives, and less frequently, astrocytoma, ependymoma, meningioma, rhabdomyosarcoma, pheochromocytoma, myeloid leukemia, and others (6).

Despite a complete disease penetrance for mutation carriers, expressivity is highly variable, spanning in severity from hundreds of tumors per patient to individuals who display no or very few tumors (7-9). This variability has triggered a number of studies on the effects of hormonal, environmental, and genetic modifiers in NF1, and a major role for genes other than the *NF1* gene in disease expressivity using quantitative and binary traits for scoring was demonstrated (10).

The high frequency of neurofibromas in some patients with NF1 together with a heterogenous phenotype suggest an increased somatic mutation rate (high second hit frequency) in a subpopulation of patients, and in subsets of cells of the same patient. An increased somatic mutation rate in cellular subpopulations would be indicated by somatic mosaicism of NF1. Mosaic expression of mutated NF1 has been described (11-17).

Taken together, it appears that genetic modification in the *NF1* gene itself is not sufficient to explain the variability of NF1 symptoms. Here we propose that mitochondria (mt) variability is a major candidate for modification of the clinical tumor phenotype in NF1. Mitochondria mutation, proliferation and structural aberrations are the cause for a number of diseases with variable and heterogeneous manifestation (18). Alteration of mitochondrial function and adaptation has recently been implied in tumorigenesis and apoptosis. A number of studies have linked mtDNA polymorphism and mutations to cell apoptosis in leukemia and lipomatosis, and amplification of mtDNA in oncocytic tumors. Recently, somatic mutations of mtDNA have been identified in human colorectal cancer, breast and other cancers (for references see attached manuscript,19). Importantly, it has been shown that mitochondria associate with neurofibromin (20), and with microtubules which are highly dependent on mitochondrial energy metabolism.

Mitochondria generate cellular energy in the form of ATP by oxidative phosphorylation (OXPHOS) and thus they are essential components for eucaryotic cells. Three aspects of mitochondrial OXPHOS are of potential importance for tumorigenesis: (i) energy production, (ii) generation of reactive oxygen species (ROS), and (iii) regulation of programmed cell death, or apoptosis. Most cells contain hundreds of mitochondria and each mitochondrion contains between 1 and 100 mt genomes. Mitochondrial DNA features a high mutation rate which, together with its multiplicity, accounts for the often perplexing phenotypes observed. When a mutation in mtDNA arises, cells initially contain a mixture of wild type (wt) and mutant mtDNA (heteroplasmy). Replicative segregation leads to homoplasmy in which each cell contains only a single mitochondrial subclass. Cells in which a threshold proportion of mutated mitochondria is reached are functionally impaired.

There is now ample evidence of mitochondrial involvement in increasing cancer risk due to oxidative stress, DNA damage, apoptosis regulation (18). In this project we investigated the possibility that mitochondrial components are involved in the pathogenesis of NF1 and that this component is a major contribution for the observed variability. This study consists of two components:

In aim 1 we study the correlation between the genotype of mitochondrial DNA (mtDNA) and the clinical phenotype in NF1. A cohort of NF1 patients will be recruited for this study and falls in two groups: A group with a high tumor burden and a group with low tumor burden. Altogether, 400 patients and 75 controls will be needed to find a meaningful correlation between mtDNA variation and tumor burden. The mtDNA will be analyzed for 20 known variable sites.

In aim 2 we study somatic mutations in mitochondrial DNA in tumors of NF1 patients. Altered mitochondria may have a significant replicative advantage in affected tissue and might thus be amplified in tumors. Certain mtDNA mutations might thus promoter tumor development. In this aim cutaneous and plexiform neurofibromas were resected from NF1 patients and the entire mtDNA analyzed and compared with paired blood mtDNA from the same patients.

Body

Aim 1: We finished recruiting patients to this study. At this point, 412 patients have been recruited, which fall at a ratio of 159 – NF1 patients with a low number of cutaneous and neurofibromas and 251 patients with a high number of cutaneous neurofibromas, respectively. In addition, DNA samples from 75 healthy control subjects have been collected.

From all the recruited patients blood samples were obtained and DNA was isolated. All samples have been tested for mtDNA variation. The other samples are currently being analyzed.

Preliminary analysis of the data revealed that NF1 patients with a low number of dermal neurofibromas carry more mitochondrial polymorphisms than patients with a high number of neurofibromas (Table 1). A consistent correlation between certain types of polymorphisms with neurofibroma burden is not statistically significant with the numbers of patients tested.

In this study, only mtDNA polymorhisms and mutations were tested that have been described to be relevant in other diseases. This list excluded the D-loop region. However, our data show that D-loop mutations may be relevant for NF1 (see below). It may, therefore, be useful to test the DNA from the NF1 cohort for polymorphisms in the D-loop.

Aim 2: We have recruited 19 NF1 patients with plexiform neurofibromas and 20 NF1 patients with cutaneous neurofibromas for this part of the study. We obtained 1 tumor sample and a paired blood sample from each of the NF1 patients with plexiform nfs. We obtained 2 or more tumors and a paired blood sample from each of the NF1 patients with cutaneous neurofibromas. From three of the patients with cutaneous neurofibromas two additional unaffected skin samples were obtained, along with the cutaneous neurofibromas and blood samples.

DNA was isolated from all samples. Except for 1 patient with plexiform neurofibromas, all DNAs were of sufficient quality to analyze the entire mitochondrial genome for mutations by temporal temperature gradient electrophoresis (TTGE).

Mutational analysis detected somatic mtDNA mutations in 9 of the 18 plexiform neurofibromas and in 5 of 13 cutaneous neurofibromas. All mutations occurred in the hypervariable D-loop regions. Most tumors were homoplasmic or nearly homoplasmic for the

mutated mtDNA, indicating accumulation of the mutated mitochondria and supporting our initial hypothesis.

A surprising finding was the homoplasmic state of mutated mitochondria in many of these mixed cell tumors. In addition, separate tumors from the same patient all harbored the same mitochondria genotype. This indicates that either all tumor cells derive from a single stem cell with a certain mitochondria genotype present, or normal cells in the body harbor a pre-existing mutated mitochondria. To analyze the second hypothesis, we analyzed unaffected skin samples and cutaneous neurofibromas. Mutated mitochondria were readily detected in these samples at a heteroplasmic state. Taken together, our data show that mitochondrial mutation pre-exist in normal tissues of NF1 patients and accumulate in tumors, suggesting a selective advantage for the mutated mitochondria in tall cells of he tumors.

This finding is supported by the analysis of mtDNA mutations in cells microdissected from neurofibromas (Schwann cells, fibroblasts) and normal tissues (endothelial cells, skin epithelial cells and dermal fibroblasts). This analysis revealed that neurofibroma-derived Schwann cells are homoplasmic for the mutated mtDNA. Normal endothelial cells and epitheliel cells are homoplasmic for the germ-line mtDNA. Fibroblasts from normal skin and from neurofibromas, however, show an increasing proportion of mutated mtDNA in the tumors.

Key Research Accomplishments

There are three research accomplishments I wish to point out:

1. We have established for the first time that plexiform and cutaneous neurofibromas in NF1 patients harbor somatic mitochondrial DNA mutations. That these mutations are found in most all different cells of the tumors (Schwann cells, fibroblasts), that the mutated mitochondria accumulate in the tumor cells and that all mutations occur in the D-loop region. These findings suggest a promoting raises the question on the function of these mutations for neurofibroma growth.
2. We have shown for the first time that somatic mitochondrial DNA mutations exist even in unaffected normal tissues (dermal fibroblasts) in NF1 patients. These mutated mitochondria are present together with normal mitochondria in a heteroplasmic state. We have also shown that these mutated mitochondria accumulate in tumor tissue, eliminating the normal (germ line) mitochondria from the tumor cells. This indicates a selective advantage for the mutated mitochondria in tumor cells.
3. There is no correlation between age, gender or clinical severity of NF1 patients and the proportion of somatic mtDNA mutations in their tumors.
4. We have established a large data base for NF1 patients together with accompanying blood DNA samples. The data base contains anonymized information about the clinical phenotype of the patients, especially their tumor burden.
5. We have found a significant correlation between the general degree of mtDNA polymorphisms and the number of cutaneous neurofibromas in NF1 patients. A lower number of neurofibromas is associated with a higher degree of polymorphism (Table 1).

Table 1: Association between the number of dermal neurofibromas (nfs) and mtDNA polymorphisms. 25 mtDNA loci have been analyzed from 412 NF1 patients and 75 healthy, age, race and gender-matched controls.

Average Number of cutaneous nfs / subject	Number of patients in cohort	Mean number of mtDNA Polymorphism	% of subjects with >1 polymorphism	% of subjects with 0 polymorphism
303,8 (NF1)	412	1,16	25,3	38,7
0 (control)	75	1.21	23,9	36,5
1271 (NF1)	**112**	**0,85 (p=0.021)**	**18,36**	**52,8**
4,3 (NF1)	**138**	**1,13**	**36,5**	**26,9**

Reportable Outcomes

Reportable are our findings on somatic mtDNA mutations in cutaneous and plexiform neurofibromas as outlined above and in the appendix (submitted manuscript). The data have also been presented at the AACR meeting (San Francisco, 2002) and the Neurofibromatosis meeting (Aspen, 2002, Berlin, 2003).

A manuscript outlining the association results between number of cutaneous neurofibromas and mtDNA polymorphisms is in preparation.

Conclusions

We have recruited to date about 412 NF1 patients and 75 control subjects for this study and established a anonymized data base with clinical data. All of the NF1 patient and control DNAs have been analyzed. We showed that there is a statistically significant association between a low number of cutaneous neurofibromas and an increased number of mitochondrial polymorphisms.

We have confirmed our initial hypothesis that mutated mitochondria accumulate in neurofibromas, indicating a functional role for neurofibroma development and growth. It remains to be shown what kind of functional consequences the mutations found (D-loop region) might have on a cellular level.

We have surprisingly found that somatic mtDNA mutations pre-exist in normal tissues of NF1 patients and that the mutated mitochondria accumulate in neurofibromas. We have defined the cell types (Fibroblasts and Schwann cells, but not endothelial cells) within the tumors that carry mtDNA mutations.

Our data support the hypothesis that cells that form cutaneous neurofibromas occur early in development, disseminate and form multiple tumors. The accumulation of mtDNA mutations may promote tumor growth.

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Appendices

Submitted manuscript 'Somatic mitochondrial DNA mutations in Neurofibromatosis Type 1 associated Tumors' by Andreas Kurtz, Maria Lueth, Lan Kluwe, Rosemary Foster, Victor-Felix Mautner, Melanie Hartmann, Robert L. Martuza, Satatoshi Sakuma, Duan-Jun Tan, Reinhard E. Friedrich, Pablo Hernaiz Driever, Lee-Jun C. Wong.

Somatic mitochondrial DNA mutations in Neurofibromatosis type 1-associated tumors

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The abbreviations used are:

CSB	conserved sequence block
LOH	loss of heterozygosity
mtDNA	mitochondrial DNA
MSI	microsatellite instability
np	nuleotide position
ND2	NADH Dehydrogenase Subunit 2
NF1	neurofibromatosis type 1
OXPHOS	oxidative phosphorylation
PAH	phenylalanine hydroxylase
PCR	polymerase chain reaction

PNS	peripheral nervous system
SCA 1	spinocerebellar ataxia type 1
SCA 3	spinocerebellar ataxia type 3
TTGE	temporal temperature gradient gel electrophoresis

Abstract

Neurofibromatosis type 1 (NF1) is an autosomal dominantly inherited disease predisposing to a multitude of tumors, most characteristically benign plexiform neurofibromas and diffuse cutaneous neurofibromas. Although penetrance is 100%, expressivity is highly variable. To test the possibility that somatic mitochondrial DNA (mtDNA) mutations may modify tumor development in NF1, we investigated the presence of somatic mtDNA mutations in neurofibromas, and traced these mutations to distinct cutaneous neurofibromas and unaffected tissue. MtDNA alterations in the entire mitochondrial genome were analyzed by temporal temperature gradient gel electrophoresis (TTGE) followed by DNA sequencing. Somatic mtDNA mutations in tumors were found in 7 out of 19 individuals with cutaneous neurofibromas and in 9 out of 18 patients with plexiform neurofibromas. A total of 34 somatic mtDNA mutations were found. Several plexiform neurofibromas from individual patients had multiple homoplasmic mtDNA mutations. In cutaneous neurofibromas, the same mtDNA mutations were always found in tumors from different locations of the same individual. An increase in the proportion of identical mutant mtDNA was demonstrated in the distinct cutaneous neurofibromas when compared to unaffected tissues. The domination by an identical mtDNA mutation in all distinct tumors of the same individual indicates a common cellular origin of the multiple neurofibromas, and a replicative advantage rather than random segregation for cells carrying these mutated mitochondria.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominantly inherited disorder with an estimated prevalence range from 1/2190 to 1/7800 (1). There are no known ethnic groups in which NF1 does not occur or is unusually common. Clinical features of NF1 include café-au-lait spots, axillary freckling, iris hamartomas (Lisch nodules), skeletal abnormalities, and learning deficiencies (2). The most prevalent clinical manifestations are the development of benign plexiform and cutaneous neurofibromas. Plexiform neurofibromas can progress at low frequency to highly malignant peripheral nerve sheath tumors (MPNSTs). Cutaneous neurofibromas typically start to develop around puberty, and the number of these tumors increases with age. Other tumor types less frequently associated with NF1 include optic gliomas, myeloid leukemias, phaeochromocytomas, and astrocytomas.

Mutations in the *NF1* tumor suppressor gene are the underlying cause for the complete disease penetrance but there is marked variability of the disease phenotype, which is not completely explained by the type of mutation in the *NF1* gene (3-7). Loss of heterozygosity (LOH) at the *NF1* locus has been shown in Schwann cells of cutaneous and plexiform neurofibromas and in MPNSTs, suggesting that the *NF1* gene product, neurofibromin, normally functions as a tumor suppressor (8-11). The tumor suppressor function likely is related to neurofibromin's *ras*-GTPase activating (rasGAP) function and deregulation of *ras* signaling (2,12-14).

The development of a plethora of mostly benign tumors in NF1, for some of which LOH for *NF1* was not found (15), and the variable expressivity of the tumor phenotype suggest that besides *NF1* – haploinsufficency, generalized reduction of the

threshold for tumorigenesis is caused by additional modulators (16,17). Here we analyze mitochondrial DNA (mtDNA) mutations as possible modulators in tumorigenesis in benign neurofibromas. In addition, characterization of somatic mtDNA mutations in multiple tumors of the same patient may illuminate the common cellular origins of multiple tumors in NF1.

The role of mitochondria in tumor development has gained much attention with recent reports of somatic mitochondrial DNA (mtDNA) mutations in brain, ovarian, esophageal, breast, and colorectal human cancers (18-26). Mitochondria contain multiple copies of circular double stranded DNA molecules that have a high degree of sequence variations among different individuals (27). In addition to energy production by oxidative phosphorylation (OXPHOS), mitochondria play a crucial role in programmed cell death (28-31) and generate reactive DNA damaging oxygen species (ROS) as side products of normal function. MtDNA is an easy target for oxidative DNA damage due to the close proximity to ROS – production, the lack of protective histone proteins, and limited repair capabilities. The accumulation of ROS might also contribute to increased nuclear gene mutagenesis (32).

The characteristics of multisystemic manifestation, variable expressivity, somatic mosaicism of NF1, as well as the reported associations of neurofibromin with highly energy dependent microtubules (33) and mitochondria (34), prompt us to hypothesize that mtDNA variations and/or somatic mtDNA mutations modulate the diffuse clinical expression of NF1. We report the presence of mtDNA alterations in neurofibromas and their dynamics between non-tumor tissues and cutaneous and plexiform neurofibromas

Materials and Methods

Tissue Samples

Patients with NF1 were recruited through the Departments of Neurosurgery and Neurogenetics, Massachusetts General Hospital, Harvard University, and through the Department of Neurology, Klinikum Nord Ochsenzoll, Hamburg, Germany, according to institutional review board approved protocols. Patients were phenotypically characterized for features of NF1, including number, location, and size of cutaneous neurofibromas. Only patients with a clear diagnosis of NF1 according to NIH criteria (35) were included in this study. Cutaneous and plexiform neurofibromas were removed during routine surgery, dissected into multiple aliquots, and frozen immediately. Two or more cutaneous neurofibromas resected from different anatomical sites on each individual were obtained from 19 patients. For three of these patients, skin samples were biopsied from an area overlaying the resected cutaneous neurofibroma and from an area distal to the tumor (Table 2, patients 4, 5 and 6). A single plexiform neurofibroma sample was obtained from each of eighteen patients.

The age of patients with cutaneous tumors ranges from 16 to 50 with a mean age of 37.6. The age of patients with plexiform tumors ranges from 8 to 73 with a mean age of 28.8.

DNA Isolation

DNA was isolated from frozen tissues using proteinase K and phenol/chloroform extraction method. DNA was extracted from peripheral blood lymphocytes using a modified non-enzymatic method (36). Total DNA was quantified using fluorescent

Hoechst dye H33258 with DYNA QUANT 200 according to manufacturer's protocol and diluted to 5ng/µl to be used in PCR reactions (37).

Mutational Analysis of the Entire Mitochondrial Genome

DNA isolated from 19 pairs of matched blood and cutaneous neurofibroma samples and from 18 pairs of matched blood and plexiform neurofibroma samples was used for mutational analysis of the mitochondrial genome by temporal temperature gradient gel electrophoresis (TTGE).

Thirty-two pairs of overlapping primers were used to amplify the entire mitochondrial genome by PCR (37). The PCR amplified DNA fragments vary from 306 bp to 805 bp in length. The positions and the sequence of the PCR primers, and the PCR and TTGE conditions were as recently described (37). Briefly, the DNA template, after the initial denaturation at 94°C for 5 min, was amplified with 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, and completed by 4 min of extension at 72°C. The PCR products were denatured at 95 °C for 30 sec and slowly cooled to 45°C for a period of 45 min at a rate of 1.1°C/min. The reannealed homoduplexes and heteroduplexes were maintained at 4 °C until TTGE analysis was performed on a Bio-Rad D-Code apparatus. Five microliters of denatured and reannealed PCR products were loaded onto a polyacrylamide gel (acrylamide: bis 37.5:1) prepared in 2X TAE buffer containing 6 M urea. Electrophoresis was carried out at 145 V for 4-5 hours at a constant 1-2°C/hour temperature increment (36). The temperature range was determined by computer simulation from the melting curve of the analyzed DNA fragment (MacMelt software, Bio-Rad Laboratories). The gels were

stained with 2 mg/L ethidium bromide for 5 min and imaged with a digital CCD gel documentation system (High performance ultra-violet transilluminator, Ultra-Violet Products).

On TTGE analysis, a single band shift represents a homoplasmic DNA alteration, and a multiple-banding pattern represents a heteroplasmic mutation (38). Any DNA fragments showing different banding patterns between the matched blood and tumor sample pairs were sequenced to identify the exact mutations.

Sequence Analysis

Confirmation of the nucleotide alteration was carried out by direct DNA sequencing of the purified PCR product using the original PCR primers and a BigDye terminator cycle sequencing kit (Perkin Elmer) and analyzed on ABI 377 (Applied Biosystem) automated sequencer. The results of DNA sequence analysis were compared with the published Cambridge sequence (39) using Mac Vector™ 7.0 (Oxford Molecular Ltd., Oxford, England) software. Sequence variations found in both tumor and blood mtDNA were scored as germline variations. Each was then checked against the Mitomap database (40). Variations not recorded in the database were categorized as novel mtDNA variations, and those appearing in the database were reported as polymorphisms. Any mtDNA sequence differences found between a tumor sample and its corresponding blood sample were scored as somatic mtDNA mutations specific to the tumor.

Laser capture microdissection

Microdissection was performed using a PixCell II (Arcturus) laser capture microscope according to the manufacturer's instructions. Frozen 10 μm sections were prepared on uncoated slides from cutaneous neurofibromas and unaffected skin tissues from the same patients (Table 2, patients #4 and #5). The sections were stained in hematoxylin and eosin immediately before microdissection. Adjacent control slides were stained with antibody to S100 protein to confirm histological and morphological differentiation of Schwann cells and fibroblasts within the neurofibromas. Fibroblasts, Schwann cells and endothelial cells were dissected from neurofibromas. Epithelial skin cells and dermal fibroblasts were dissected from skin samples. Approximately 200-300 clearly distinguishable cells of each type were dissected from each tissue for DNA extraction (PicoPure extraction kit, Arcturus). The D-loop region of the mtDNA was amplified by PCR and directly sequenced.

Results

Somatic mtDNA mutations in neurofibromas

MtDNA from pairs of matched tumor and normal blood samples was analyzed in parallel by TTGE using multiple PCR products comprising the entire mitochondrial genome. Parallel analysis of a single PCR product from matched blood and tumor samples allowed the rapid detection of somatic nucleotide alterations shown as differences in the banding patterns of the products. These analyses were carried out on a total of 19 patients with cutaneous neurofibromas and 18 patients with plexiform neurofibromas. Representative results are shown in Figure 1A and 1B.

In panel A, TTGE analysis of the D-loop region of mtDNA showed a band shift in plexiform neurofibroma T173 compared to its corresponding blood sample B174, suggesting the presence of a homoplasmic mutation in the tumor sample. Direct sequencing of the blood and tumor mtDNA PCR products revealed 5 homoplasmic tumor specific nucleotide substitutions in this region, including 3 novel ones, A16163G, C16186T, and C16221T. The detected T16189C and T16519C alterations have been reported previously (20,23,25).

Panel B illustrates a similar analysis of plexiform neurofibroma T191 and its corresponding blood sample B192, showing a multiple banding pattern. Sequencing of the PCR products revealed two changes from homoplasmic T199 and G207 to heteroplasmic T199C and G207A. Interestingly, a heteroplasmic change in the proportion of T204C mutation between B192 and T191 was also detected, revealing a shift in the degree of heteroplasmy at T204C in the tumor sample.

To rule out the possibility that some mutations may not be detectable by TTGE, we randomly chose 6 samples that did not show TTGE positive banding patterns and sequenced 10 coding regions containing stretches of 6-8 homopolynucleotides. No mutations were found. We believe that the somatic mtDNA mutations observed in NF1 are not due to PCR artifact or random MSI. The complete results of our analysis of NF-1 associated cutaneous and plexiform neurofibromas are listed in Table 1.

MtDNA mutations in plexiform neurofibromas

Among the 18 pairs of mtDNA from plexiform neurofibromas analyzed, 9 showed somatic mtDNA mutations (Table 1A). Four of the plexiform tumors with mtDNA mutations harbored a single alteration (4/9) while the remaining five cases (5/9) had more than 1 mutation. For example, 9 distinct somatic mtDNA mutations were found in tumor T173. A total of 27 different somatic mtDNA mutations were identified. All mutations were found in the D-loop region. Insertions or deletions in the nucleotide position (np) 303-309 poly C region were detected in multiple tumors. This region has been reported to be the somatically unstable mutation hot spot of breast cancer (23,41). All nucleotide substitutions were transitions between T/C and A/G, consistent with oxidative DNA damage. The majority (14 out of 27) of the somatic mtDNA mutations in the plexiform tumors were alterations from homoplasmic state in blood to homoplasmic state in tumor. A shift from homoplasmy in blood to heteroplasmy in the corresponding tumor was found in 6 cases, and a shift from heteroplasmy in blood to homoplasmy in tumor was detected in 4 cases. Three pairs of matched samples had heteroplasmic nucleotide substitutions in both blood and tumor but there were detectable differences in the degree of heteroplasmy as assessed by quantitative comparison of the nucleotide

peak amplitudes in the corresponding sequence profiles (Table 1A).

A plexiform tumor, T173, harbored 9 somatic mtDNA mutations when compared to its corresponding blood sample B174. One suspicion is that there may have been an error in DNA sampling. To rule out this possibility, identity analysis on samples T173 and B174 was performed. Identical alleles were detected at 5 polymorphic sites: the short tandem repeat in intron 3 of the *PAH* gene (chromosome 12), the CTG repeats of the myotonin protein kinase gene (disease gene for myotonic dystrophy, chromosome 19), the CAG repeats of the androgen receptor gene (X chromosome), and in the *SCA 1* (chromosome 6) and *SCA 3* (chromosome 4) genes (data not shown). These results support that tumor T173 indeed had 9 somatic mtDNA mutations, four of which are novel.

Somatic mtDNA mutations in distinct cutaneous neurofibromas are identical

We analyzed cutaneous neurofibromas from a total of 19 patients. MtDNA from two distinct cutaneous tumors of an individual were analyzed against blood mtDNA of the same individual. Seven out of 19 patients with cutaneous neurofibromas had somatic mtDNA mutations in their tumors (Table 1B), and all of them occurred in the hypervariable D-loop region.

Surprisingly, separate tumors from single NF1 patients harbored always the same somatic mtDNA mutation, as was the case for tumor samples T104 and T105 (Figure 1, panel C), revealing a change in a short poly C sequence at np 303-309 in the conserved sequence block (CSB) which shifted from C8/C9 heteroplasmy in the blood sample (B106) to near homoplasmy of C8 in both tumor samples. A similar progressive

shift in the percentage of heteroplasmy was observed in other sets of tumors. These samples comprise 8 sets of two independent tumors resected from distinct anatomical sites on a single individual.

Blood mtDNA from patient 1 (Table 2, B106) is heteroplasmic for 303-309 C8/C9. Both of its tumors T105 and T104 showed apparently homoplasmic C8, although sequence analysis may not be able to reveal difference in very low percentage of heteroplasmy. Analysis of mtDNA samples from tumors T107 and T108 showed a shift from np 303-309 C7 homoplasmy in the matched blood mtDNA sample B109 to C7/C8 heteroplasmy in each of the tumors. Tumors T119 and T120 both harbor the same homoplasmic T16304C mutation when compared to the corresponding blood DNA B121, which is homoplasmic for the wild type T16304. Comparative changes were detected in tumors and blood of patients 4, 5 and 6 (Table 2).

Tumor tissues from different parts of the same tumor also showed the identical mtDNA mutations with comparable degrees of heteroplasmy throughout the tumor (Table 2, patients 4, 5). In patient 7, we detected a high proportion of np 303-309 C8 in blood and tumor samples, while in patient 8 no D-loop mutations were found.

Somatic mtDNA mutations are present in normal skin of NF1 patients

NF1 patients usually have multiple cutaneous neurofibromas throughout the PNS. These are complex tumors composed predominantly of Schwann cells and fibroblasts, with a minority of neurons, macrophages and endothelial cells. As noted above, homoplasmy for somatic mtDNA mutations was detected in a number of these cutaneous neurofibromas of mixed cell type (Table 1B, tumor samples T104, T105,

T119, and T120). Additionally, multiple cutaneous neurofibromas resected from distinct anatomical sites on an affected individual shared identical somatic mtDNA mutations (Tables 1B and 2).

These results suggest that the tumor-specific mtDNA mutation profile may have already been present in the relevant cell types outside of tumors and accumulate in the tumor. To examine this possibility, we analyzed mtDNA isolated from unaffected skin and matched cutaneous neurofibromas obtained from NF1 patients. We focused our mtDNA somatic mutation analysis of these skin and tumor samples on the D-loop and its surrounding region since the previous data obtained from studying 19 cutaneous and 18 plexiform neurofibromas revealed that all the somatic mtDNA mutations occurred in the D-loop region. The results of our analyses are shown in Table 2 (patients 4, 5, and 8).

One of these sets (patient 8) did not show any somatic mtDNA D-loop mutations in the tumor and skin samples (Table 2). The other two sets of tumors displayed somatic mtDNA mutations (patients 4 and 5). A progressive increase in mutant mtDNA content was demonstrated between blood, skin distal from and overlaying the tumor, and the neurofibroma in both of these patients (Table 2). In addition, the identical mutation was present in the skin, in different parts of the tumor, and in separate tumors from different locations of the same individual.

MtDNA mutations are cell restricted

To determine which cell type in an apparently homoplasmic cutaneous neurofibroma contains the mutated mitochondria, mutational analysis was performed on

laser capture microdissected cells from cutaneous neurofibromas. Schwann cells, fibroblasts and endothelial cells were dissected from neurofibromas 583, 584 (patient 4) and 592 and 593 (patient 5) (Table 2). Fibroblasts and epithelial cells were also dissected from unaffected skin of the same patients (586, 588, Table 2). MtDNA from the separate cell types was analyzed for the presence of the previously identified D-loop mutation 303-309 insC C7/C8.

In tumors 583 and 584, Schwann cells and fibroblasts contained the C8 mtDNA mutation (>90%) (Figure 2A-F). Tumor derived endothelial cells contained only C7 mtDNA (Figure 2G-I), as did epithelial cells obtained from the skin sample 586 of the same patient (Figure 2J). Dermal fibroblasts from skin sample 586 contained C7 and C8 mtDNA at a ratio of about 30:70 (Figure 2K). Similar results were obtained for the different cell types of tumor samples 592 and 593, and matched skin sample 588. The results of the cell type specific mtDNA analysis are summarized in Table 3.

Correlation of somatic mtDNA mutations with clinical features

The association between the presence of mtDNA mutations in plexiform neurofibromas and the phenotype of the affected individuals revealed no sex, age or clinical difference between the individuals who did or did not have somatic mtDNA alterations (Table 4). One point worth noting is that tumor T173 with 9 somatic mtDNA mutations arose in the youngest patient (age 8) from this study.

Germline Sequence Variations

In the analyses of somatic mtDNA mutations in NF1-associated tumors, several

sequence variations in multiple blood mtDNA samples were detected (Table 1). When the mtDNA sequences from blood was compared with that of the published Cambridge sequence (http://www.mitomap.org), numerous germline sequence variations were revealed (Table 5). A total of 71 distinct germ-line variations were identified from the sequenced fragments. These do not represent all the sequence variations since only the mtDNA PCR products of regions that showed somatic mutations by TTGE in the paired tumor sample were sequenced. Nine of the germline variations detected are novel, and the remaining have been reported in the Mitomap database. Many of the mtDNA germline variations reported here occurred in multiple individuals. Among them, A73G and T16519C are common polymorphisms while A263G and 303-315insC represent polymorphisms in the Cambridge sequence (39,43). Although germline variations are generally considered silent, missense mutations such as the novel A265V alteration in the mitochondrial electron transport chain complex subunit ND2 may have a functional effect.

Discussion

This is the first comprehensive mutational analysis of the entire mitochondrial genome to demonstrate that somatic mtDNA mutations are present in cutaneous and plexiform neurofibromas associated with NF1. A total of 18 distinct mtDNA alterations were detected in the analyzed tumors. Five of these mutations are novel, whereas the remaining 13 mutations have been reported as somatic mtDNA alterations in other tumor types (Table 1). The percentage of neurofibromas with somatic mtDNA mutations is similar to those found in glioblastoma and medulloblastoma, but lower than those in lung, breast, and oral cancers (18-26).

All of the mtDNA somatic mutations identified in our study occured in the hypervariable D loop region of the mitochondrial genome. This is unique since numerous studies on lung, breast, ovarian, bladder, head and neck, glioblastoma, and oral cancers showed that 20-70% of somatic mtDNA mutations were found in coding regions (Table 6). The pathological significance of mutations in non-coding regions of the mitochondrial genome is currently unknown. It is possible that mutations in the conserved sequence block, the origin of replication, and the promoter region may affect the mitochondrial biogenesis, transcription and protein expression (44,45).

One surprising finding was the identification of 9 distinct somatic homoplasmic mtDNA mutations in a single plexiform neurofibroma (T173). The cause for this unusually large number of tumor specific somatic mtDNA mutations is not clear. It is possible that the point mutations in the origin of H-strand replication and the termination-associated sequence regulate the mtDNA synthesis and transcription in the tumor. A large number (>6) of somatic mtDNA mutations also occurred in approximately

5-10% of medulloblastomas, breast, and lung cancers (23,26). As outlined in Table 6, there is an average of about 1-3 somatic mtDNA mutations per tumor (18,20,23,25). In the analysis of NF1-associated tumors, one single somatic mtDNA mutation was detected in each cutaneous neurofibroma, but an average of three mutations was detected per plexiform neurofibroma. It is noteworthy that 22 out of 27 mutations in plexiform neurofibromas are nucleotide substitutions, compared to only 2 out of 7 mutations in cutaneous neurofibromas. One interesting observation is that mutations at np204 and np207 occurred 3 times in 3 unrelated patients. This result suggests that the np204 and np207 are either mutation hot spots or the mutant mitochondria have selective growth advantage.

The most common somatic mtDNA mutations identified in our study are insertions or deletions at 303-309. Microsatellite instability (MSI) was not detected in any of the other 10 short tandem repeat regions in the mitochondrial genome (data not shown). The mutations at np303-309 region were not observed in mtDNA from 40 muscle tissues of individuals ranging in age from 0 to 65 years (unpublished observation). This suggests that the variability in the np303-309 region is due to a mutational hotspot rather than a true MSI. It is of interest to note that the np303-309 C8 mutation always accumulated in cutaneous neurofibromas, while the C7 variant present in blood in homoplasmic or C7/C8 heteroplasmic state was either diminished or not detected in tumors.

MtDNA mutations may occur randomly, and multiple mutations may or may not occur simultaneously. In order to reach a homoplasmic state, there must be some mechanism for advantageous selection and/or a sufficient number of cell divisions.

Mutations in the origin of replication (D-loop region) may provide a replicative advantage of these mutant mtDNAs. This is supported by the observation that multiple mutations in tumors of the same patient were almost always found in a homoplasmic state. Furthermore, the same homoplasmic mutations were found in distinct cutaneous neurofibromas resected from different anatomical sites of a single individual. The independent appearance of the same mtDNA mutation in distinct tumors of the same patient in 6 analyzed cases strongly argues against a random process, which may also cause homoplasmy of mtDNA mutations in tumors, a sufficient number of cell divisions provided (47). The genetic background of cells has also been shown to influence the dynamics of mitochondrial segregation and maintenance (48). Since neurofibromin is closely associated with microtubules and mitochondria (33,34), haploinsufficiency of *NF1* may perhaps similarly affect the stability and segregation of mitochondria.

The presence of the identical homoplasmic somatic mtDNA mutation in distinct cutaneous neurofibromas from a single individual, make the independent occurrence of the same mutation in the distant tumors unlikely. Instead, the observations indicate that mtDNA alterations in cells exist before the development of tumors. We readily detected heteroplasmic mtDNA mutations in the D-loop region in fibroblasts of unaffected tissue confirming this hypothesis. Furthermore, the progressive increase in the prevalence of the mutant mitochondria, with lowest proportions in skin samples and highest in any of the distinct tumors of the same individual supports the selective growth of cells carrying these mtDNA mutations. Indeed, Schwann cells and fibroblasts from cutaneous neurofibromas are homoplasmic for the mutated mtDNA.

Taken together, somatic mtDNA mutations are present in apparently normal cells in NF1 and may thus occur early in development. Mutated mitochondria are enriched in multiple discrete tumors in NF1 patients, suggesting that they provide a functional advantage for tumorigenesis in the diffuse pattern observed clinically.

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Figure Legend

Figure 1. Detection of somatic mtDNA mutations in plexiform and cutaneous neurofibromas by TTGE and sequence analysis.

A, Comparison of PCR amplified mtDNA D-loop region from plexiform neurofibroma T173 and paired blood sample B174. Sequencing revealed multiple homoplasmic nucleotide substitutions in plexiform neurofibroma mtDNA.

B, Comparison of mtDNA D-loop region from plexiform neurofibroma T191 and paired blood sample B192. Sequencing revealed two changes T199C and G207A, from homoplasmic in normal to heteroplasmic in tumor and one heteroplasmic to heteroplasmic T204C change in the same region.

C, Comparison of mtDNA D-loop region from two cutaneous neurofibromas T104 and T105 from the same individual and paired blood sample B106. Sequencing revealed a gradual change from a heteroplasmic 303-309 C8/C9 to a homoplasmic C8 in both cutaneous neurofibromas.

Figure 2. Cell specificity of mtDNA mutations in cutaneous neurofibromas and skin of the same patient.

A-C, Cutaneous neurofibroma 583 with selected Schwann cells (A), microdissected cells (B) and sequence results of np303-309 C7/C8 D-loop region (C). Arrow: C8.

D-F, The same neurofibroma as in (A) with selected fibroblasts (D), microdissected fibroblasts (E) and sequence results of np303-309 D-loop region (F). Arrow: C8.

G-I, The same neurofibroma as in (A) with selected endothelial cells (G), microdissected cells (H) and sequencing results (I). Arrow: C7. Magnification is 200x in all images.

J, Sequencing results of epithelial cells from a skin sample of the same patients as in (A). Arrow: C7.

K, Sequencing results of dermal fibroblasts from a skin sample of the same patient as in (A). Arrow: C8 (70%)/C7(30%).

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Table 1 A *somatic mtDNA mutations in NF1 associated plexiform neurofibromas*

Case number	Gene/ region	Somatic Mutation	Cambridge Sequence	nl to tu Pattern[a]	Function[c]	Previously reported in tumors[b]	Reference
T159	D-LOOP	A73G[c]	A	homo-homo	Hypervariable Segment 2	eso	22
T159	D-LOOP	C16193T	C	homo-homo	Hypervariable Segment 1	novel	this study
T159	D-LOOP	C16278T	T	homo-homo	Hypervariable Segment 1	ov	18
T159	D-LOOP	C16519T	T	homo-homo		lung, glioblastoma	20,25
T165	D-LOOP	T64C	C	hetero-hetero	Hypervariable Segment 2	novel	this study
T171	D-LOOP	303-309delC,C9/8-C8/9	C_7	hetero-hetero	CSB[d]	crc,gastric,eso,ov, brca	18,19,22,32
T173	D-LOOP	C64T	C	homo-homo	Hypervariable Segment 2	novel	this study
T173	D-LOOP	A73G	A	homo-homo	Hypervariable Segment 2	eso	22
T173	D-LOOP	T152C	T	homo-homo	H-strand origin	ov	18
T173	D-LOOP	T195C	T	homo-homo	H-strand origin	lung, glioblastoma	20,25
T173	D-LOOP	A16163G	A	homo-homo	Termin.associated sequ.[e]	novel	this study
T173	D-LOOP	C16186T	C	homo-homo	7S DNA	novel	this study
T173	D-LOOP	T16189C	T	homo-homo	7S DNA	brca	18
T173	D-LOOP	C16221T	C	homo-homo	Hypervariable Segment 1	novel	this study
T173	D-LOOP	T16519C	T	homo-homo		lung, glioblastoma	20,25
T179	D-LOOP	303-309insC,C7/8-C8	C_7	hetero-homo	CSB	crc,gastric,eso,ov, brca	18,19,22,32
T185	D-LOOP	C204T	T	homo-homo	H-strand origin	gastric, glioblastoma	19,25
T185	D-LOOP	A207G	G	hetero-homo	H-strand origin	brca	18
T187	D-LOOP	303-309delC,C7/8-C7	C_7	hetero-homo	CSB	crc,gastric,eso,ov, brca	18,19,22,32
T189	D-LOOP	T204C	T	hetero-homo	H-strand origin	gastric, glioblastoma	19,25
T189	D-LOOP	G207A	G	homo-hetero	H-strand origin	brca	18
T189	D-LOOP	303-309insC,C7-C7/8	C_7	homo-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,32
T191	D-LOOP	A73G	A	homo-hetero	Hypervariable Segment 2	eso	22
T191	D-LOOP	T199C	T	homo-hetero	Hypervariable Segment 2	ov	18
T191	D-LOOP	T204C	T	hetero-hetero	H-strand origin	gastric, glioblastoma	19,25
T191	D-LOOP	G207A	G	homo-hetero	H-strand origin	brca	18
T191	D-LOOP	303-309insC,C7-C7/8	C_7	homo-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,32

[a] nl: normal (blood); tu: tumor; homo: homoplasmic; hetero: heteroplasmic
[b] crc: colorectal cancer; eso: esophageal cancer; ov: ovarian cancer; brca: breast cancer
[c] The letter before the np-number indicates the nucleotide found in the normal tissue. The letter after the np-number indicates the nucleotide found in tumor tissue. The homoplasmic or heteroplasmic status of the mutation in normal and tumor tissue is indicated accordingly in column 'nl to tu pattern'.
[d] CSB: conserved sequence block
[e] Termin.: Termination associated sequence;

Table 1 B *Somatic mtDNA mutations in NF1 associated cutaneous neurofibromas*

Case number	Gene/ region	Somatic Mutation	Cambridge Sequence	nl to tu Pattern[a]	Function	Previously reported in tumors[b]	Reference
T104	D-LOOP	303-309delC,C9/8-C8	C_7	hetero-homo	CSB[c]	crc,gastric,eso,ov, brca	18,19,22,31
T105	D-LOOP	303-309delC,C9/8-C8	C_7	hetero-homo	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T107	D-LOOP	303-309insC,C7-C7/8	C_7	homo-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T108	D-LOOP	303-309insC,C7-C7/8	C_7	homo-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T119	D-LOOP	T16304C	T	homo-homo	Hypervariable Segment 1	ov	18
T120	D-LOOP	T16304C	T	homo-homo	Hypervariable Segment 1	ov	18
T590	D-LOOP	303-309insC,C7/8-C8/7	C_7	hetero-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T591	D-LOOP	303-309insC,C7/8-C8/7	C_7	hetero-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T583	D-LOOP	303-309insC,C7/8-C8/7	C_7	hetero-homo	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T584	D-LOOP	303-309insC,C7/8-C8/7	C_7	hetero-homo	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T468	D-LOOP	T196C	T	hetero-hetero	CSB	novel	
T469	D-LOOP	T196C	T	hetero-homo	CSB	novel	
T554	D-LOOP	303-309insC,C7/8-C8/7	C_7	hetero-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,31
T555	D-LOOP	303-309insC,C7/8-C8/7	C_7	hetero-hetero	CSB	crc,gastric,eso,ov, brca	18,19,22,31

[a]nl: normal (blood); tu: tumor; homo: homoplasmic; hetero: heteroplasmic
[b]crc: colorectal cancer; eso: esophageal cancer; ov: ovarian cancer; brca: breast cancer
[c]CSB: conserved sequence block

Table 2 ***Somatic mtDNA mutations in two separate cutaneous neurofibromas[a].***

Patient #	sample #	Specimen type	Location	Somatic mutation	% of heteroplasmy[b]	
1						
	106	blood		303-309 insC C7/C8	C7~50%	C8~50%
	105	tumor 1		303-309 delC C7/C8	C7~0%	C8~100%
	104	tumor 2		303-309 delC C7/C8	C7~5%	C8~95%
2						
	109	blood		303-309 insC C7/C8	C7~100%	C8~0%
	108	tumor 1		303-309 insC C7/C8	C7~60%	C8~40%
	107	tumor 2		303-309 insC C7/C8	C7~40%	C8~60%
3						
	121	blood		T16304	T~100%	C~0%
	120	tumor 1		T16304C	T~0%	C~100%
	119	tumor 2		T16304C	T~0%	C~100%
4						
	587	blood		303-309 insC C7/C8	C7~50%	C8~50%
	586	skin	distal from tumor	303-309 insC C7/C8	C7~40%	C8~60%
	585	skin	overlaying the tumor	303-309 insC C7/C8	C7~30%	C8~70%
	583	tumor 1	Thorax/Abdomen	303-309 insC C7/C8	C7~10%	C8~90%
	584	tumor 2	Thorax/Abdomen	303-309 insC C7/C8	C7~5%	C8~95%
5						
	594	blood		303-309 insC C7/C8	C7~50%	C8~50%
	588	skin	distal from tumor	303-309 insC C7/C8	C7~40%	C8~60%
	589	skin	overlaying the tumor	303-309 insC C7/C8	C7~40%	C8~60%
	590	tumor 1	Thorax right side	303-309 insC C7/C8	C7~20%	C8~80%
	591	tumor 2, part 1	Thorax/Abdomen	303-309 insC C7/C8	C7~0%	C8~100%
	592	tumor 2, part 2	Thorax/Abdomen	303-309 insC C7/C8	C7~20%	C8~80%
	593	tumor 2, part 3	Thorax/Abdomen	303-309 insC C7/C8	C7~0%	C8~100%
6						
	276	blood		T196C	T~50%	C~50%
	468	tumor 1		T196C	T~30%	C~70%
	469	tumor 2		T196C	T~5%	C~95%
7						
	9	blood		303-309 insC C8/C9	C8~70%	C9~30%
	554	tumor		303-309 insC C8/C9	C8~95%	C9~5%
	555	tumor		303-309 insC C8/C9	C8~70%	C9~30%
8						
	598	blood				
	597	skin	2cm away from 595	no mutation found		
	596	tumor 1	Thorax right side	no mutation found		
	595	tumor 2	Thorax right side	no mutation found		

[a] Somatic mtDNA mutations in two separate cutaneous neurofibromas from each of 8 patients, and in paired skin samples of patients 4, 5, 8. In all samples from patients 4-8 only D-loop region was analyzed.
[b] Percentage of heteroplasmy was estimated from the sequencing chromatogram. They do not represent the actual proportion. However, the trend of progressive alteration was obvious (patients, 4 and 5). For samples 104 and 105 TTGE gel chromatogram was used to estimate the percentage of mutant heteroplasmy, which was too low to be revealed by sequencing (Fig. 1C).

Table 3 ***Somatic mtDNA mutations in cells dissected from cutaneous neurofibromas and paired skin of patients #4 and #5 of table 2.***

Patient #	sample #	Specimen type	Cell type	Somatic mutation	% of heteroplasmy[a]	
4						
	586	skin	epithelial	303-309 insC C7/C8	C7~100%	C8~0%
	586	skin	dermal fibroblast	303-309 insC C7/C8	C7~30%	C8~70%
	583	tumor 1	Schwann cell	303-309 insC C7/C8	C7~0%	C8~100%
	583	tumor 1	fibroblast	303-309 insC C7/C8	C7~5%	C8~95%
	583	tumor 1	endothelial cell	303-309 insC C7/C8	C7~100%	C8~0%
5						
	588	skin	epithelial	303-309 insC C7/C8	C7~100%	C8~0%
	588	skin	dermal fibroblast	303-309 insC C7/C8	C7~35%	C8~65%
	592	tumor 2, part 2	Schwann cell	303-309 insC C7/C8	C7~5%	C8~95%
	592	tumor 2, part 2	fibroblast	303-309 insC C7/C8	C7~10%	C8~90%
	592	tumor 2, part 2	endothelial cell	303-309 insC C7/C8	C7~100%	C8~0%

[a] Percentage of heteroplasmy was estimated from the sequencing chromatogram.

Table 4*Clinical Information about patients with plexiform neurofibromas*[a]

Case #	sex	Age y	known *NF* mutation	cutaneous Neurofibromas	subcutaneous Neurofibromas	plexiform Neurofibromas	café-au-lait spots	axillary freckling	groin freckling	Lisch nodules
T159	**f**	**39**	**yes**	**35**	**0**	**1**	**>9**	**bilateral**	**no**	**yes**
T165	**m**	**17**	**no**	**0**	**0**	**2**	**>12**	**bilateral**	**bilateral**	**yes**
T171	**f**	**24**	**yes**	**0**	**0**	**2**	**>12**	**bilateral**	**bilateral**	**yes**
T173	**m**	**8**	**no**	**0**	**5**	**1**	**9**	**no**	**no**	**yes**
T179	**f**	**11**	**yes**	**10 to 50**	**0**	**1**	**>12**	**bilateral**	**bilateral**	**no**
T185	**m**	**63**	**yes**	**>300**	**0**	**1**	**4**	**no inf.**	**no inf.**	**yes**
T187	**m**	**13**	**yes**	**<10**	**0**	**1**	**>6**	**bilateral**	**bilateral**	**yes**
T189	**f**	**13**	**yes**	**0**	**<10**	**2**	**>6**	**bilateral**	**bilateral**	**yes**
T191	**m**	**73**	**no**	**20**	**0**	**3**	**>12**	**bilateral**	**bilateral**	**yes**
T157	f	10	no	0	0	1	>6	bilateral	no	no
T161	m	34	no	>200	30	1	>6	bilateral	bilateral	yes
T163	m	57	no	>300	0	2	>12	bilateral	no	yes
T169	f	11	no	0	2	1	>12	bilateral	no	yes
T175	m	45	no	>300	>50	2	>12	bilateral	bilateral	yes
T177	m	34	no	>2000	0	1	>6	bilateral	bilateral	yes
T181	f	38	yes	<10	0	1	>12	bilateral	bilateral	yes
T183	m	24	yes	<10	0	1	>12	no inf.	no inf.	no inf.
T193	m	29	yes	0	0	1	>6	no inf.	no inf.	no inf.

[a] patients with somatic mtDNA mutations are in bold

Table 5 *Germline sequence variations* [a,b]

A. Novel Gene/region	Germ-line mutation	Frequency[c]	Significance
D-loop	T10C	1	7S DNA
D-loop	T55C	1	7S DNA
D-loop	T57C	1	Hypervariable Segment 2
D-loop	T408A	1	L-strand promoter
16S	A2706G	1	16S RNA
ND2	C5263T	1	GCC-GTC, **A265V**
COI	G6917A	1	GTG-GGG, V338V
ND4	A11947G	1	ACA-ACG, T396T
D-loop	C16465T	1	

B.Reported Gene/region	Germ-line mutation	Frequency[c]	Significance
D-loop	T72C	4	Hypervariable Segment 2
D-loop	A73G	12	Hypervariable Segment 2
D-loop	T146C	2	H-strand origin
D-loop	C150T	1	H-strand origin
D-loop	T152C	4	H-strand origin
D-loop	A189G	1	H-strand origin
D-loop	C194T	2	H-strand origin
D-loop	T195C	6	H-strand origin
D-loop	T199C	1	H-strand origin
D-loop	T204C	2	H-strand origin
D-loop	G207A	2	H-strand origin
D-loop	C242T	1	mtTF1 binding site
D-loop	A263G	16	H-strand origin
D-loop	C271T	1	H-strand origin
D-loop	C295T	1	mtTF1 binding site
D-loop	303-309insC	15	CSB II
D-loop	C462T	1	
D-loop	T489C	1	
D-loop	A508G	1	
D-loop	514insCA	1	
D-loop	514insCACA	1	
D-loop	568insCCC	1	
12s	A663G	2	12S RNA
12s	G709A	1	12S RNA
ND2	G4580A	1	ATG-ATA, M37M
ND2	A4769G	2	ATA-ATG, M100M
COI	T6776C	1	CAT-CAC, H291H
ND4	G11914A	1	ACG-ACA, T385T

Table 5 *Continued*

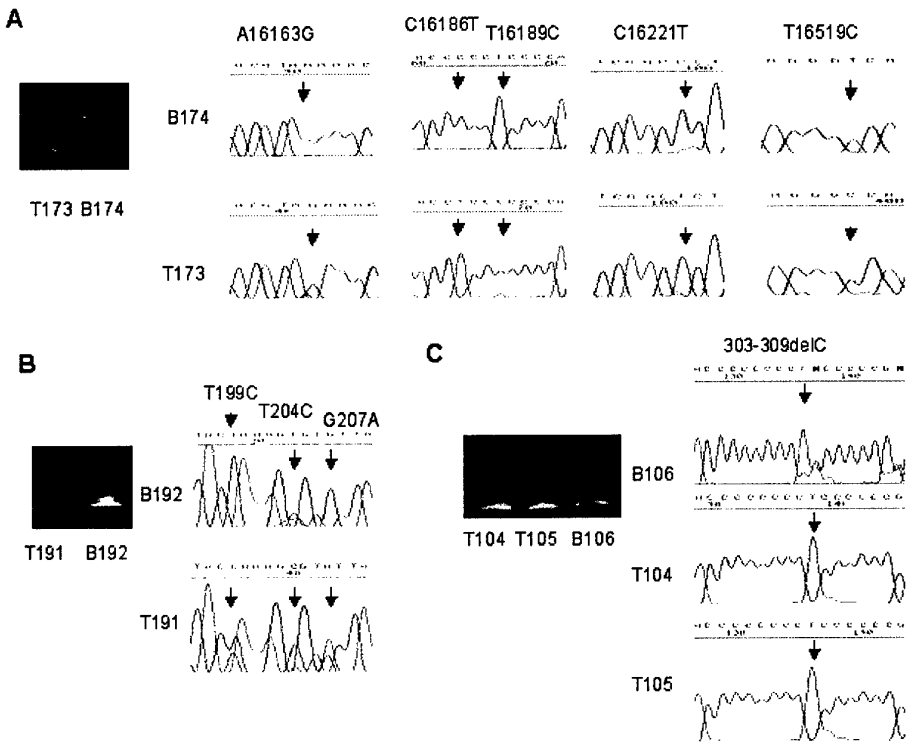
B.Reported Gene/region	Germ-line mutation	Frequency[c]	Significance
ND4	G12007A	1	TGG-TGA, W416W
ND5	A12612G	1	GTA-GTG, V92V
ND5	A12693G	1	AAA-AAG, K119K
ND5	C12705T	2	ATC-ATT, I123I
ND6	A14233G	1	ATC-GTC, **I29V**
CytB	T14798C	1	TTC-CTC, **F18L**
D-loop	G16145A	1	
D-loop	C16186T	1	Hypervariable Segment 1
D-loop	C16188T	1	Hypervariable Segment 1
D-loop	T16189C	1	Hypervariable Segment 1
D-loop	T16192T	1	Hypervariable Segment 1
D-loop	C16193T	1	Hypervariable Segment 1
D-loop	C16195T	1	Hypervariable Segment 1
D-loop	C16222T	1	Hypervariable Segment 1
D-loop	C16223T	3	Hypervariable Segment 1
D-loop	C16278T	4	Hypervariable Segment 1
D-loop	C16290T	1	Hypervariable Segment 1
D-loop	C16292T	1	Hypervariable Segment 1
D-loop	C16294T	3	Hypervariable Segment 1
D-loop	C16296T	2	Hypervariable Segment 1
D-loop	T16298C	3	Hypervariable Segment 1
D-loop	T16304C	2	Hypervariable Segment 1
D-loop	A16309G	1	Hypervariable Segment 1
D-loop	T16311C	2	Hypervariable Segment 1
D-loop	T16362C	2	Hypervariable Segment 1
D-loop	G16390A	1	Hypervariable Segment 1
D-loop	T16519C	4	

[a] Total number of distinct germline sequence variations: 71
Novel: 9
Reported: 62
[b] Missense substitutions are in bold
[c] Number of tumors, which carry germline-variation

Table 6 ***Summary of mtDNA mutations in various tumors***

	Our studies						Other studies				
	NF1 cutaneous	plexiform	lung	breast	Medullo-blastoma	oral	ovarian	bladder	Head & neck	Glio-blastoma	lung
No. of tumors	16	18	14	19	10	18	10	14	13	17	14
No. of cases with mtDNA mutations	7	9	10	14	5	14	6	9	6	6	6
% tumor with mtDNA mutation	43	50	71	74	50	78	60	64	46	35	43
Total no. of mtDNA mutations	7	27	26	27	17	27	6	20	9	18	9
Number of mutations/tumor	1	3	2.6	1.93	3.4	1.93	1	2.2	1.5	3	1.5
No. of mutations in D-loop	7	27	17	22	11	19	2	6	6	10	6
% of mutations in D-loop	100	100	65	81	65	70	33	30	67	56	67
Reference	this study	this study	to be published	25	28	51	20	22	22	27	17

Figure 1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C16221T
T16519C
B174
T173 B174
T173
B
T199C
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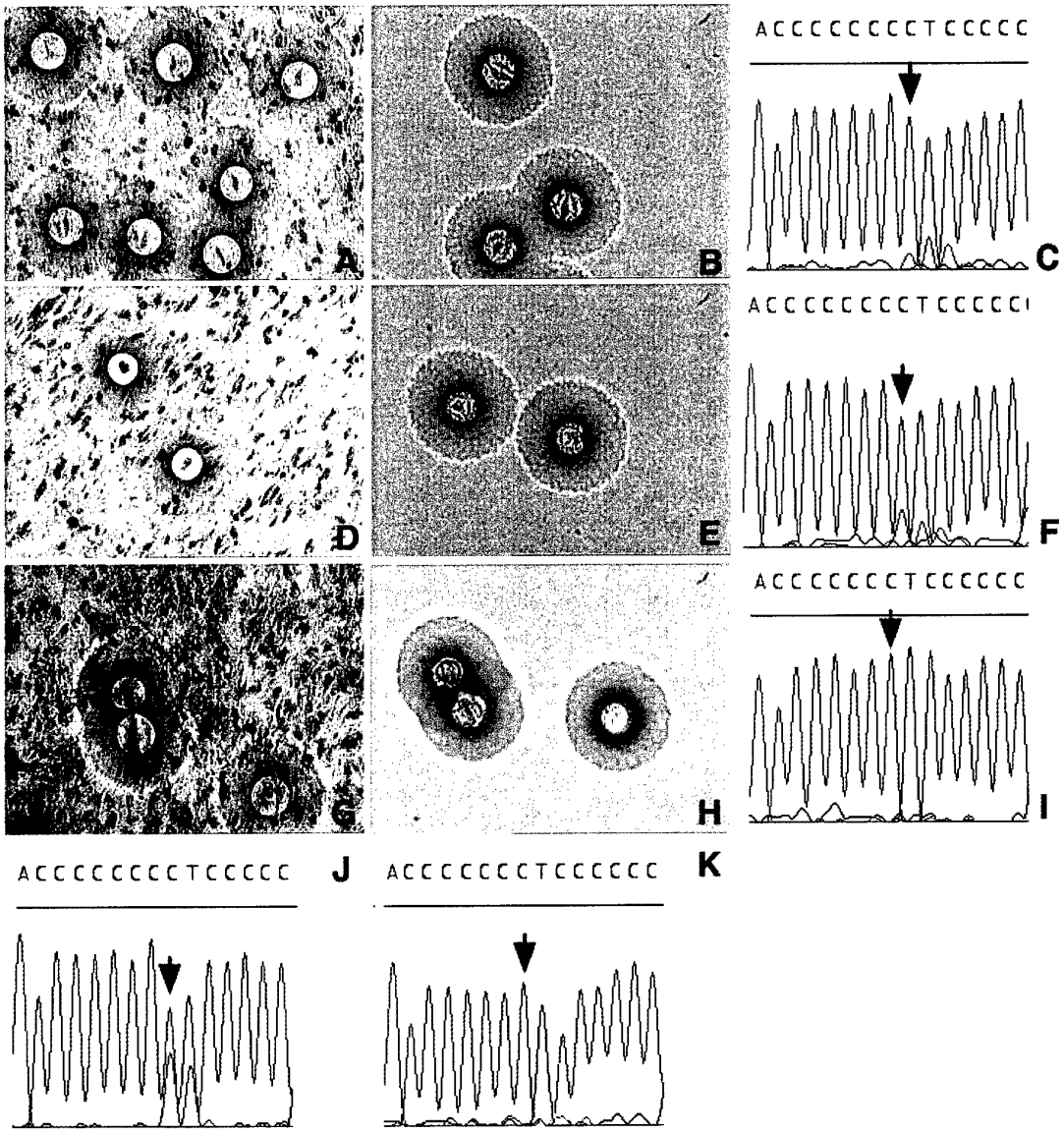
B192
T191 B192
T191
C
303-309delC
B106
T104 T105 B106
T104
T105

Figure 2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D
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G
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K